BLADDER NUCLEAR MATRIX PROTEINS, POLYNUCLEOTIDE SEQUENCES ENCODING THEM, AND THEIR USE

5   The present application is a continuation-in-part of co-pending application Serial No. 08/742,850, filed on November 1, 1996.

BACKGROUND OF THE INVENTION

10   The present invention relates generally to bladder nuclear matrix proteins, called "NMPs" here, and more specifically to novel nuclear matrix proteins of the bladder which are associated with cell-proliferative

15   disorders.

         The early diagnosis of bladder cancer is central to the effective treatment of the disease. Currently, there are no methods available to easily and specifically identify the presence of bladder cancer

20   cells.   The prevailing technique for diagnosis of bladder cancer is to identify bladder cancer cells by morphological examination of the cells by a pathologist. A cellular hallmark of the transformed phenotype is abnormal nuclear shape, the presence of multiple

25   nucleoli and altered patterns of chromatin organization. Nuclear structural alterations are so prevalent in cancer cells that they are commonly used as a

pathological marker of transformation for many types of cancer. Nuclear shape is determined in part by the nuclear matrix, the dynamic skeleton of the nucleus.

The nuclear matrix is the structural component of the nucleus that determines nuclear morphology, organizes the DNA in a three-dimensional fashion that is tissue specific, and has a central role in the regulation of a number of nuclear processes including the regulation of gene expression. The nuclear matrix has been demonstrated to play a central role in the regulation of important cellular processes such as DNA replication and transcription. Getzenberg, *J. Cell Biochem.* 55: 22-31 (1994). The nuclear matrix is the framework or scaffolding of the nucleus and consists of the peripheral laminas and pore complexes, an internal ribonucleic protein network, and residual nucleoli. Berezney *et al.*, *Biochem. Biophys. Res. Comm.* 60: 1410-17 (1974). The nuclear matrix consists of approximately 10% of the nuclear proteins and is virtually devoid of lipids, DNA and histones. Fey *et al.*, *Critical Reviews in Eukaryotic Gene Expression* 1: 127-44 (1991).

A majority of the known NMPs are common to all cell types and physiologic states. A number of laboratories have identified NMPs which may be unique to certain cell types or states. Mitogenic stimulation and the induction of differentiation have been demonstrated to alter the composition of nuclear matrix proteins and structure. The nuclear matrix contains a number of associated proteins that have been demonstrated to be involved in transformation. Berezney first showed that the nuclear matrix is altered in transformation, examining hepatoma nuclear matrix proteins. Berezney *et*

al., Cancer Res. 39: 3031-39 (1979). Fey and Penman demonstrated that tumor promoters induce a specific morphologic signature in the nuclear matrix-intermediate filament scaffold of kidney cells. Fey *et al., Proc.*

5   *Nat'l Acad. Sci. USA* 81: 859-66 (1984). Fey and Penman went on to demonstrate that the pattern of NMPs differed between normal and tumorigenic cell lines. Fey *et al., loc. cit.* 85: 121-25 (1989). Recently, an antibody to a nuclear matrix protein, termed NM-200.4, was raised from

10   the breast carcinoma cell line T-47D. Weidner *et al., Am. J. Path.* 138: 1293-98 (1991). This antibody reacts strongly with human breast carcinoma specimens as well as specimens from lung, thyroid, and ovarian cancers, but does not react with normal epithelial cells of

15   similar origin, raising the possibility of the use of certain anti-NMP antibodies as diagnostic tools.

In co-pending application Serial No. 08/015,624, the entire contents of which are incorporated by reference herein, it has been demonstrated when human

20   prostate samples were examined, nuclear matrix proteins were identified that (1) were present only in the normal prostate and were missing in both prostate cancer and benign prostatic hyperplasia (BPH) (normal pattern), (2) were found only in the prostate cancer cells and missing

25   in the normal prostate and BPH (prostate cancer pattern), and (3) were found in both normal and BPH samples but were absent from prostate cancers.

No nuclear matrix proteins have been identified heretofore, however, that are linked specifically to

30   bladder cancer.

Currently, the only available method for bladder cancer detection is morphological examination of

cytology samples or cystoscopic biopsies. This method is accurate for high grade lesions; however, a significant proportion of bladder tumors (25-45%) are low grade or well differentiated and escape detection upon cytologic examination of exfoliated cells. Cytologic examination is unable to detect low numbers of tumor cells and is unable to separate low grade or well differentiated cells from their normal counterparts. The diagnostic accuracy of cytology alone for the detection of low grade transitional carcinoma is between 49 and 64 percent. Repeating the study can increase the accuracy of cytology; however, this is a costly and time consuming practice for both the patient and physician. When bladder cancer is detected early at a localized stage, the five year survival rate is 94%. Disease that has spread regionally or distantly lowers survival to 49% and 6% respectively. Development of a sensitive screening assay that could specifically detect bladder carcinoma would significantly facilitate patient management and allow the earlier treatment of this disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of a BLCA-4 urine assay carried out with an anti-BLCA-4 antibody of the present invention on a group of normal patients and a group with bladder cancer.

Fig. 2 shows the results of a BLCA-4 urine assay carried out with an anti-BLCA-4 antibody of the present invention on a group of patients with spinal cord injury.

SUMMARY OF THE INVENTION

The present invention relates to nuclear matrix proteins that are able to differentiate cancerous bladder cells from normal bladder cells, polynucleotide sequences encoding them, and their methods of use. Six proteins, respectively designated BLCA-1, BLCA-2, BLCA-3, BLCA-4, BLCA-5 and BLCA-6, have been discovered that are present in all cancerous bladder cells that are not present in the normal bladder cells, and three proteins (referred to as BLNL-1, BLNL-2, and BLNL-3) have been discovered that are unique to normal bladder tissue. These proteins are useful for diagnosing and producing treatments for cell proliferative disorders of the bladder.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

5   According to one aspect, the present invention is directed to a purified nuclear matrix protein or a fragment thereof, which is present in normal bladder cells but absent in cancerous bladder cells, or which is absent in normal bladder cells but present in cancerous

10   bladder cells.   In particular, the present invention relates to a protein that is present in normal bladder cells but absent in cancerous bladder cells selected from the group consisting of BLNL-1, BLNL-2, and BLNL-3. In addition, the present invention relates to a protein

15   that is absent in normal bladder cells but present in cancerous bladder cells selected from the group consisting of BLCA-1, BLCA-2, BLCA-3, BLCA-4, BLCA-5, and BLCA-6.

       Another embodiment of the present invention is a

20   purified polynucleotide sequence encoding the above identified NMPs or NMP fragments.   Another embodiment is a purified polynucleotide sequence which hybridizes to the polynucleotide sequence encoding the above-mentioned

25   NMPs or NMP fragments.

       Another embodiment is a host cell transformed with a polynucleotide sequence encoding the above-mentioned NMPs or NMP fragments.   Transformation of a host cell with recombinant DNA may be carried out by conventional

30   techniques known in the art.   Where the host is prokaryotic, such as *E. coli*, competent cells which are capable of DNA uptake can be prepared from cells

harvested after the exponential growth phase and subsequently treated by the $CaCl_2$ method by procedures well known in the art. Alternatively, $MgCl_2$ or RbCl can be used. Transformation can also be performed after

5 forming a protoplast of the host cell or by electroporation.

When the host is a eukaryote, such methods of transfection of DNA as calcium phosphate co-precipitates, conventional mechanical procedures such as

10 microinjection, electroporation, insertion of a plasmid encased in liposomes, or virus vectors may be used. Eukaryotic cells can also be cotransformed with DNA sequences encoding the NMPs of the invention, and a second foreign DNA molecule encoding a selectable

15 phenotype, such as the herpes simplex thymidine kinase gene. Another method is to use a eukaryotic viral vector, such as simian virus 40 (SV40) or bovine papilloma virus, to transiently infect or transform eukaryotic cells and express the protein. EUKARYOTIC

20 VIRAL VECTORS Gluzman (ed.), Cold Spring Harbor Laboratory, 1982.

Isolation and purification of the NMPs or NMP fragments expressed by a transformed host may be carried out by conventional means including preparative

25 chromatography and immunological separations involving monoclonal or polyclonal antibodies. Antibodies provided in the present invention are immunoreactive with NMP polypeptide or fragments thereof.

Another embodiment is a recombinant expression

30 vector containing the above-mentioned polynucleotide sequences. Preferably, the vector is a virus. Preferred viruses are RNA viruses and preferred RNA

viruses are retroviruses. Another preferred vector is a
liposome, preferably a target-specific liposome which
may be targeted with, for example, an antibody or
ligand. Another preferred vector is a plasmid.

5   Another embodiment is an antibody which binds to
the above-mentioned NMPs or NMP fragments. The antibody
may be polyclonal or monoclonal.   Using the NMPs
isolated by the inventors, antibodies have been
prepared which are capable of differentiating between
10   cancerous bladder tissue and normal bladder tissue.
One embodiment of the present invention is directed to
these antibodies.   Furthermore, another embodiment of
the present invention is directed to antibodies which
can detect individuals at risk of developing bladder
15   cancer (before morphological change is even visible in
tissue samples).

     An additional embodiment of the present invention
is directed to antibodies that specifically bind to a
nuclear matrix protein or an antigen thereof according
20   to the present invention, which is advantageous over
prior art antibodies for bladder cancer because the
NMPs of the present invention that are associated with
bladder cancer cells (i.e., BLCA-1 through 6) are
believed not to be present in subjects afflicted with
25   cystitis who do not have bladder cancer. As a result,
cystitis patients who do not suffer from bladder cancer
are not falsely indicated as having cancer when
antibodies according to the present invention are used.
This is an important improvement over the prior art
30   diagnostic methods. Preferably, an antibody according
to this embodiment specifically binds to BLCA-4. Still
more preferably, an antibody according to this

embodiment specifically binds to an antigen including the amino acid sequence of SEQ ID NO: 2.

Another embodiment is a method for detecting a cell proliferative disorder in a subject or for detecting individuals at risk of developing a cell proliferative disorder, preferably bladder cancer, comprising contacting a cellular component from the subject with an antibody or nucleic acid probe which binds to a cellular component associated with the cell proliferative disorder. Preferably, the cellular component is taken from the subject's bladder and is preferably a nucleic acid. Preferably, the nucleic acid is DNA encoding the above-mentioned NMPs or NMP fragments. Also preferred as a nucleic acid is RNA. Another preferred cellular component is the above-mentioned NMPs or NMP fragments.

Preferably, the nucleic acid probe specifically hybridizes to the above-mentioned cellular component. When the reagent is a nucleic acid probe, it is preferably detectably labeled. Preferred labels include a radioisotope, a bioluminescent compound, a chemiluminescent compound, a fluorescent compound, a metal chelate, and an enzyme.

Alternatively, if the cellular component is an NMP or NMP fragment, then an antibody is used which specifically binds to the NMP or NMP fragment. As noted above, the antibody may be monoclonal or polyclonal.

Another embodiment is a method of treating a cell proliferative disorder associated with a protein selected from the group consisting of BLCA-1, BLCA-2, BLCA-3, BLCA-4, BLCA-5, BLCA-6, BLNL-1, BLNL-2, and BLNL-3, comprising administering to a subject with the

disorder a therapeutically effective amount of an antisense polynucleotide sequence that blocks the sequences encoding the above-mentioned NMPs. In this embodiment, the treatment is designed to block the

5 expression of one or more NMPs which give rise to the cell proliferative disorder.

In an alternative method of treatment, instead of using an antisense polynucleotide sequence, a polynucleotide sequence is used which encodes one of the

10 above-mentioned NMPs. In this embodiment, the treatment is designed to provide the subject with one or more NMPs that prevent or ameliorate the cell proliferative disorder.

In another method of treatment, an antibody is

15 administered to the subject which is capable of blocking the function of one or more of the above NMPs.

Another embodiment is a method of gene therapy, comprising introducing into the cells of a host subject an expression vector comprising a polynucleotide

20 sequence encoding one or more of the above-mentioned NMPs. Preferably, the expression vector is introduced into the cells of the host subject *ex vivo*, yielding transformed cells, and the transformed cells then are reintroduced into the subject. A preferred expression

25 vector for this purpose is an RNA virus, preferably a retrovirus.

Another embodiment of the present invention relates to a method for identifying a composition which blocks or enhances the function of a bladder cell NMP.

30 The inventive method comprises:

(a) incubating NMP-containing bladder cells with a test composition under conditions that

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allow the bladder cells and test composition
to interact,

and then

    (b)   measuring whether the test composition blocks

5   or enhances the function of the bladder cell
          NMP.

      Another embodiment is a kit for detecting a cell-
proliferative disorder of the bladder comprising a
nucleic acid probe that binds to a polynucleotide

10   sequence encoding one of the above-mentioned NMPs.
Preferably, the probe is labeled for ease of detection
with a label as described above. Alternatively, the kit
may comprise an antibody which specifically binds to one
of the above-mentioned NMPs. Still another alternative

15   is to use an oligonucleotide primer in the kit that
permits amplification of a target polynucleotide
sequence encoding one of the above-mentioned NMPs, for
example, by polymerase chain reaction (PCR)
amplification.

20   The NMPs of the invention include fragments and
conservatively substituted variants thereof. Minor
modifications of the NMP primary amino acid sequence may
result in proteins which have substantially equivalent
activity as compared to the NMP polypeptide described

25   herein. Such modifications may be deliberate, as by
site-directed mutagenesis, or may be spontaneous. Such
modifications include deletion of non-essential amino
acids. All of the polypeptides produced by these
modifications are included herein as long as the

30   biological activity of the native NMP still exists.
Further, deletion of one or more amino acids can also
result in a modification of the structure of the

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resultant molecule without significantly altering its biological activity. This can lead to the development of a smaller active molecule which would have broader utility.

5   The term "conservative substitution" as used herein denotes the replacement of an amino acid residue by a structurally similar residue. Examples of conservative substitutions include the substitution of one hydrophobic residue such as isoleucine, valine,

10 leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, and the like.

15 Peptides of the invention can be synthesized by the well known solid phase peptide synthesis methods described, for example, by Merrifield, *J. Am. Chem. Soc.* 85: 2149 (1962), and by Stewart and Young, SOLID PHASE PEPTIDES SYNTHESIS 27-62 (Freeman Publ., 1969).

20 The polyclonal and monoclonal antibodies of the invention are immunoreactive with the NMPs or immuno-genic fragments of the NMPs. If desired, polyclonal antibodies can be further purified, for example, by binding to and elution from a matrix to which NMP

25 polypeptide is bound or by utilizing common nuclear matrix proteins to selectively remove non-specific antibodies. Antibody which consists essentially of pooled monoclonal antibodies with different epitopic specificities, as well as distinct monoclonal antibody

30 preparations are provided. The term "antibody" as used in this invention includes intact molecules as well as fragments thereof, such as Fab and $F(ab')_2$ fragments,

which are functionally capable of binding an epitopic determinant of an NMP.

A preferred method for the identification and isolation of antibody binding domains which exhibit

5   binding with NMP is the bacteriophage λ vector system. This vector system has been used to express a combinatorial library of Fab fragments from the mouse antibody repertoire in *Escherichia coli*, see Huse *et al.*, *Science* 246: 1275-81 (1989), and from the human antibody

10 repertoire. Mullinax *et al.*, *Proc. Nat'l Acad. Sci. USA* 87: 8095-99 (1990).

As used herein, the term "cell-proliferative disorder" denotes malignant as well as non-malignant (or benign) disorders of the bladder. This term further

15 encompasses hyperplastic disorders of the bladder. The cells comprising these proliferative disorders often appear morphologically and genotypically to differ from the surrounding normal tissue. As noted above, cell-proliferative disorders may be associated, for example,

20 with expression or absence of expression of the NMPs of the invention. Expression of an NMP at an inappropriate time during the cell cycle or in an incorrect cell type may result in a cell-proliferative disorder. The NMP-encoding polynucleotide in the form of an antisense

25 polynucleotide is useful in treating hyperplasia and malignancies of the bladder. When the cell-proliferative disorder is associated with NMP expression, (*e.g.*, BLCA-1, 2, 3, 4, 5 and/or 6), an antisense NMP polynucleotide sequence or NMP binding

30 antibody can be introduced into the bladder cells to block the expression and/or function of the NMP. Alternatively, when the cell proliferative disorder is

associated with under-expression or expression of a mutant NMP polypeptide (e.g., BLNL 1-3), a polynucleotide sequence encoding the missing or under-expressed NMP can be introduced into the cell.

5   For purposes of the invention, an antibody or nucleic acid probe specific for an NMP may be used to detect the presence of the NMP polypeptide (in the case of an antibody probe) or polynucleotide (in the case of the nucleic acid probe) in biological fluids or tissues

10   suspected of containing the NMP. Oligonucleotide primers based on any coding sequence region in the NMP sequence are useful for amplifying DNA or RNA, for example by PCR. Any specimen containing a detectable amount of antigen can be used. A preferred sample of

15   this invention is tissue taken from the bladder. Alternatively, biological fluids which may contain cells of the bladder may be used.

        The term "subject" as used herein refers to mammals, preferably humans.

20   Another technique which may also result in greater sensitivity consists of coupling the probe to low molecular weight haptens. These haptens can then be specifically detected by means of a second reaction. For example, it is common to use such haptens as biotin,

25   which reacts with avidin, or dinitrophenol, pyridoxal, and fluorescein, which can react with specific antihapten antibodies.

        The method for detecting a cell expressing a particular NMP of the invention or a cell-proliferative

30   disorder associated with an NMP, described above, can be utilized for detection of residual bladder cancer or other malignancies or benign hyperplasia conditions in a

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subject in a state of clinical remission. Additionally, the method for detecting NMP polypeptide in cells is useful for detecting a cell-proliferative disorder by identifying cells expressing specific NMPs in comparison

5   with NMPs expressed in normal cells. Using the method of the invention, NMP expression can be identified in a cell and the appropriate course of treatment can be employed (e.g., NMP-encoding or antisense gene therapy, as well as conventional chemotherapy). Since the

10 expression pattern of the NMPs of the invention vary with the stage of malignancy of a cell, a sample of bladder tissue can be screened with a panel of NMP-specific reagents (e.g., nucleic acid probes or antibodies to NMPs) to detect NMP expression and

15 diagnose the stage of malignancy of the cell.

The monoclonal antibodies of the invention are suitable for use in immunoassays in which they can be utilized in liquid phase or bound to a solid phase carrier. In addition, the monoclonal antibodies in

20 these immunoassays can be detectably labeled in various ways. Examples of types of immunoassays which can utilize monoclonal antibodies of the invention are competitive and non-competitive immunoassays in either a direct or indirect format. Examples of such

25 immunoassays are the radioimmunoassay (RIA) and the sandwich (immunometric) assay. Detection of the antigens using the monoclonal antibodies of the invention can be performed utilizing immunoassays which are run in either the forward, reverse, or simultaneous

30 modes, including immunohistochemical assays on physiological samples. Alternatively, the antibody of the invention can be used to detect NMPs present in

electrophoretically dispersed gel protocols such as Western blots and two-dimensional gels.

The monoclonal antibodies of the invention can be bound to many different carriers and used to detect the

5   presence of NMP. Examples of well-known carriers include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, natural and modified celluloses, polyacrylamides, agaroses and magnetite. The nature of the carrier can be either soluble or

10   insoluble for purposes of the invention.

In performing the assays it may be desirable to include certain "blockers" in the incubation medium (usually added with the labeled soluble antibody). The "blockers" are added to assure that non-specific

15   proteins, proteases, or anti-heterophilic immunoglobulins to anti-NMP immunoglobulins present in the experimental sample do not cross-link or destroy the antibodies on the solid phase support, or the radiolabeled indicator antibody, to yield false positive

20   or false negative results. The selection of "blockers" therefore may add substantially to the specificity of the assays described in the present invention.

It has been found that a number of nonrelevant (i.e., nonspecific) antibodies of the same class or

25   subclass (isotype) as those used in the assays (e.g., IgG1, IgG2a, IgM, etc.) can be used as "blockers." The "blockers" are used at a level high enough to maintain the proper sensitivity yet inhibit any unwanted interference by mutually occurring cross reactive

30   proteins in the specimen (normally 1-100 $\mu g/\mu l$).

In this description, the term "epitope" denotes any determinant capable of specific interaction with the

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monoclonal antibodies of the invention. Epitopic determinants usually comprise chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional

5   structural characteristics, as well as specific charge characteristics.

In using the monoclonal antibodies of the invention for the *in vivo* detection of antigen, the detectably labeled monoclonal antibody is given in a

10   dose which is diagnostically effective. The term "diagnostically effective" means that the amount of detectably labeled monoclonal antibody is administered in sufficient quantity to enable detection of the site having the NMP antigen for which the monoclonal antibody

15   is specific.

The dosage of detectably labeled monoclonal antibody for *in vivo* diagnosis will vary depending on such factors as age, sex, and extent of disease of the individual. The dosage of monoclonal antibody can vary from about

20   0.001 mg/m^2, to about 500 mg/m^2, preferably 0.1 mg/m^2 to about 200 mg/m^2, most preferably about 0.1 mg/m^2 to about 10 mg/m^2. Such dosages may vary, for example, depending on whether multiple injections are given, tumor burden, and other factors.

25   For *in vivo* diagnostic imaging, the type of detection instrument available is a major factor in selecting a given radioisotope. The radioisotope chosen must have a type of decay which is detectable for a given type of instrument. Still another important

30   factor in selecting a radioisotope for *in vivo* diagnosis is that the half-life of the radioisotope be long enough so that it is still detectable at the time of maximum

uptake by the target, but short enough so that deleterious radiation with respect to the host is minimized. Ideally, a radioisotope used for *in vivo* imaging will lack a particle emission, but produce a

5   large number of photons in the 140-250 keV range, which may be readily detected by conventional gamma cameras.

For *in vivo* diagnosis, radioisotopes may be bound to immunoglobulin either directly or indirectly by using an intermediate functional group. Intermediate

10   functional groups which often are used to bind radioisotopes which exist as metallic ions to immunoglobulins are the bifunctional chelating agents such as diethylenetriaminepentacetic acid (DTPA) and ethylenediaminetetraacetic acid (EDTA) and similar

15   molecules. Typical examples of metallic ions which can be bound to the monoclonal antibodies of the invention are ^{111}In, ^{97}Ru, ^{67}Ga, ^{68}Ga, ^{72}As, ^{89}Zr, and ^{201}Tl.

The monoclonal antibodies of the invention can also be labeled with a paramagnetic isotope for purposes

20   of *in vivo* diagnosis, as in magnetic resonance imaging (MRI) or electron spin resonance (ESR). In general, any conventional method for visualizing diagnostic imaging can be utilized. Usually gamma and positron emitting radioisotopes are used for camera imaging and para-

25   magnetic isotopes for MRI. Elements which are particularly useful in such techniques include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Cr, and ^{56}Fe.

The monoclonal antibodies of the invention can be used to monitor the course of amelioration of an NMP-

30   associated cell-proliferative disorder. Thus, by measuring the increase or decrease in the number of cells expressing a NMP or changes in NMP present in

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various body fluids, such as ejaculate or urine, it would be possible to determine whether a particular therapeutic regimen aimed at ameliorating the disorder is effective.

5   The monoclonal antibodies of the invention can also be used, alone or in combination with effector cells, see Douillard *et al.*, *Hybridoma* 5 (Supp. 1): S139 (1986), for immunotherapy in an animal having a cell proliferative disorder which expresses NMP polypeptide

10   with epitopes reactive with the monoclonal antibodies of the invention.

When used for immunotherapy, the monoclonal antibodies of the invention may be unlabeled or attached to a therapeutic agent. These agents can be coupled either

15   directly or indirectly to the monoclonal antibodies of the invention. One example of indirect coupling is by use of a spacer moiety. These spacer moieties, in turn, can be either insoluble or soluble, see Diener *et al.*, *Science* 231: 148 (1986), and can be selected to enable

20   drug release from the monoclonal antibody molecule at the target site. Examples of therapeutic agents which can be coupled to the monoclonal antibodies of the invention for immunotherapy are drugs, radioisotopes, lectins, and toxins.

25   The drugs which can be conjugated to the monoclonal antibodies of the invention include non-proteinaceous as well as proteinaceous drugs. The terms "non-proteinaceous drugs" encompasses compounds which are classically referred to as drugs, for example,

30   mitomycin C, daunorubicin, vinblastine, and others used to treat cancer.

The proteinaceous drugs with which the monoclonal

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antibodies of the invention can be joined include immunomodulators and other biological response modifiers. The term "biological response modifiers" encompasses substances which are involved in modifying

5   the immune response in such manner as to enhance the destruction of an NMP-associated tumor for which the monoclonal antibodies of the invention are specific. Examples of immune response modifiers include such compounds as lymphokines. Lymphokines include tumor

10   necrosis factor, the interleukins, lymphotoxin, macrophage activating factor, migration inhibition factor, colony stimulating factor, and interferon. Interferons with which the monoclonal antibodies of the invention can be labeled include alpha-interferon, beta-

15   interferon and gamma-interferon and their subtypes.

In using radioisotopically conjugated monoclonal antibodies of the invention for immunotherapy certain isotopes may be more preferable than others depending on such factors as tumor cell distribution as well as

20   isotope stability and emission. If desired, the tumor cell distribution can be evaluated by the *in vivo* diagnostic techniques described above. Depending on the cell proliferative disease some emitters may be preferable to others. In general, alpha and beta

25   particle-emitting radioisotopes are preferred in immunotherapy. For example, if an animal has solid tumor foci a high energy beta emitter capable of penetrating several millimeters of tissue, such as ^{90}Y, may be preferable. On the other hand, if the cell

30   proliferative disorder consists of simple target cells, as in the case of leukemia, a short range, high energy alpha emitter, such as ^{212}Bi, may be preferable.

Examples of radioisotopes which can be bound to the monoclonal antibodies of the invention for therapeutic purposes are ^{125}I, ^{131}I ^{90}Y, ^{67}Cu, ^{212}Bi, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, ^{65}Zn, and ^{188}Re.

5   Lectins are proteins, usually isolated from plant material, which bind to specific sugar moieties. Many lectins are also able to agglutinate cells and stimulate lymphocytes. Ricin is a toxic lectin which has been used immunotherapeutically. The alpha-peptide chain of

10   ricin, which is responsible for toxicity, may be bound to the antibody of the invention to enable site specific delivery of the toxic effect.

Toxins are poisonous substances produced by plants, animals, or microorganisms, that, in sufficient

15   dose, are often lethal. Diphtheria toxin is a substance produced by *Corynebacterium diphtheria* which can be used therapeutically. This toxin consists of an alpha and beta subunit which under proper conditions can be separated. The toxic A component can be bound to an

20   antibody and used for site specific delivery to a NMP bearing cell.

The monoclonal antibodies of the invention can be used in combination with alpha-interferon. This treatment modality enhances monoclonal antibody

25   targeting of carcinomas by increasing the expression of monoclonal antibody reactive antigen by the carcinoma cells. Greiner *et al.*, *Science* 235: 895 (1987). Alternatively, the monoclonal antibody of the invention can be used, for example, in combination with gamma-

30   interferon to thereby activate and increase the expression of Fc receptors by effector cells which, in turn, results in an enhanced binding of the monoclonal

antibody to the effector cell and killing of target tumor cells.

It is also possible to utilize liposomes with the monoclonal antibodies of the invention in their membrane to specifically deliver the liposome to the tumor expressing NMP. These liposomes can be produced such that they contain, in addition to the monoclonal antibody, such immunotherapeutic agents as those described above which would then be released at the tumor site. Wolff *et al.*, *Biochemical et Biophysical Acta* 802: 259 (1984).

The dosage ranges for the administration of monoclonal antibodies of the invention are those large enough to produce the desired effect in which the symptoms of the malignant disease are ameliorated. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any complication. Dosage can vary from about 0.1 mg/kg to about 2000 mg/kg, preferably about 0.1 mg/kg to about 500 mg/kg, in one or more dose administrations daily, for one or several days. Generally, when the monoclonal antibodies of the invention are administered conjugated with therapeutic agents, lower dosages, comparable to those used for *in vivo* diagnostic imaging, can be used.

The monoclonal antibodies of the invention can be administered parenterally by injection or by gradual perfusion over time. The monoclonal antibodies of the

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invention can be administered intravenously, intra-
peritoneally, intramuscularly, subcutaneously, intra-
cavity, or transdermally, alone or in combination with
effector cells.

5   The present invention also provides a method for
treating a subject with an NMP-associated cell-
proliferative disorder using an NMP nucleotide sequence.
An NMP nucleotide sequence which may encode a suppressor
polypeptide may be under-expressed as compared to
10   expression in a normal cell, therefore it is possible to
design appropriate therapeutic or diagnostic techniques
directed to this sequence.   Thus, where a cell-
proliferative disorder is associated with the expression
of an NMP associated with malignancy, nucleic acid
15   sequences that interfere with NMP expression at the
translational level can be used.   This approach
utilizes, for example, antisense nucleic acid and
ribozymes to block translation of a specific NMP mRNA,
either by masking that mRNA with an antisense nucleic
20   acid or by cleaving it with a ribozyme.   In cases when a
cell proliferative disorder or abnormal cell phenotype
is associated with the under expression of NMP
suppressor for example, nucleic acid sequences encoding
NMP (sense) could be administered to the subject with
25   the disorder.

          Antisense nucleic acids are DNA or RNA molecules
that are complementary to at least a portion of a
specific mRNA molecule. Weintaub, *Scientific American*,
262: 40 (1990).   In the cell, the antisense nucleic
30   acids hybridize to the corresponding mRNA, forming a
double-stranded molecule.   The antisense nucleic acids
interfere with the translation of the mRNA since the

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cell will not translate a mRNA that is double-stranded. Antisense oligomers of about 15 nucleotides are preferred, since they are easily synthesized and are less likely to be expressed than larger molecules when
5   introduced into the target NMP-producing cell.

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of nucleotide sequences which
10   encode these RNAs, it is possible to engineer molecules that recognize specific nucleotide sequences in an RNA molecule and cleave it. Cech, *J. Amer. Med. Assn.* 260: 3030 (1988). A major advantage of this approach is that, because they are sequence-specific, only mRNAs
15   with particular sequences are inactivated.

There are two basic types of ribozymes namely, *tetrahymena*-type (Hasselhoff, *Nature*, 334:585, 1988) and "hammerhead"-type. *Tetrahymena*-type ribozymes recognize sequences which are four bases in length, while
20   "hammerhead"-type ribozymes recognize base sequences 11-18 bases in length. The longer the recognition sequence, the greater the likelihood that that sequence will occur exclusively in the target mRNA species. Consequently, hammerhead-type ribozymes are preferable
25   to *tetrahymena*-type ribozymes for inactivating a specific mRNA species and 18-based recognition sequences are preferable to shorter recognition sequences.

The present invention also provides gene therapy for the treatment of cell proliferative disorders which
30   are mediated by NMP. Such therapy requires introduction of the appropriate NMP polynucleotide sequence (antisense or encoding strand) into cells of subjects

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having the proliferative disorder. Delivery of antisense NMP polynucleotides can be achieved using a recombinant expression vector such as a chimeric virus or a liposome. Disorders associated with under-expression of an NMP or expression of a cancer-associated NMP can be treated using gene therapy with the encoding or antisense nucleotide sequences, respectively.

Various viral vectors which can be utilized for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting an NMP sequence of interest into the viral vector along with another gene which encodes the ligand for a receptor on a specific target cell, for example, the vector is rendered target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector.

Since recombinant retroviruses are defective in one or more genes, they require assistance in order to

produce infectious vector particles. Helper cell lines which have deletions of the packaging signal include but are not limited to Ψ2, PA317 and PA12, for example. These cell lines produce empty virions, since no genome

5 is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and vector virion produced.

10 Alternatively, NIH 3T3 or other tissue culture cells can be directly transfected with plasmids encoding the retroviral structural genes *gag, pol* and *env* by conventional calcium phosphate transfection. These cells are then transfected with the vector plasmid

15 containing the genes of interest. The resulting cells release the retroviral vector into the culture medium.

Other targeted delivery systems for NMP antisense polynucleotides include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based

20 systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (ULV), which range in size from

25 0.2-4.0 μm can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form. Fraley *et al., Trends*

30 *Biochem. Sci.* 6: 77 (1981).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-

transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH,

5   ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidyl-glycerol, phosphatidylcholine, phosphatidyiserine, phosphatidylethanolamine, sphingolipids, cerebrosides,

10   and gangliosides. Particularly useful are diacyl-phosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphati-

15   dylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific and organelle-

20   specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting utilizes the natural tendency of liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries.

25   Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and

30   cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be

modified in a variety of ways. In the case of a hyposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable

5   association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

In general, the compounds bound to the surface of the targeted delivery system will be ligands and

10   receptors which will allow the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest which will bind to another compound, such as a receptor.

In general, surface membrane proteins which bind

15   to specific effector molecules are referred to as receptors. In the present invention, antibodies of the invention are preferred receptors. Antibodies can be used to target liposomes to specific cell-surface ligands, in this case the NMPs of choice. Preferably,

20   the target tissue is bladder tissue. A number of procedures can be used to covalently attach either polyclonal or monoclonal antibodies to a liposome bilayer. Antibody-targeted liposomes can include mono-clonal or polygonal antibodies or fragments thereof such

25   as Fab, or $F(ab')_2$, as long as they bind efficiently to an the antigenic epitope on the target cells.

Preparations for parenteral administration include sterile aqueous or nonaqueous solutions, suspensions, and emulsions. Examples of non aqueous solvents are

30   propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water,

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alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated

5   Ringer's intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such an those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants,

10   chelating agents and inert gases and the like.

The invention also relates to a method for preparing a medicament or pharmaceutical composition comprising the polynucleotides or the monoclonal antibodies of the invention, the medicament being used

15   for therapy of NMP associated cell proliferative disorders.

The invention is further illustrated by, though in no way limited to, the following examples.

Tissue Selection

Normal and tumor bladder tissue samples were obtained from patients undergoing surgery for bladder cancer at the University of Pittsburgh Medical Center.

5 Normal bladders were obtained from the Center for Organ Recovery and Education (CORE) via Dr. Michael J. Becich, Department of Pathology, University of Pittsburgh. These bladders provide the opportunity to study nuclear matrix composition and staining in the normal organ

10 which were compared with alterations that occur during transformation from normal to tumor. Samples were only utilized that could clearly be identified by the pathologist as containing approximately pure populations of the stated cell type.

15

Nuclear Matrix Preparation

The nuclear matrix proteins were isolated from the bladder tissue and tumors selected above as taught in Fey, *et al.*, *J. Cell Biol.*, 98:1973-1984, 1988 and

20 Getzenberg, *et al.*, *Cancer Res.*, 51:6514-6520, 1991. The tissue pieces were minced into small (1 mm^3) pieces and homogenized with a Teflon pestle on ice with 0.5% Triton X-100 in a solution containing 2 mM vanadyl ribonucleoside (RNase inhibitor) to release the lipids

25 and soluble proteins. Extracts were then filtered through a 350 micron nylon mesh and extracted with 0.25 M ammonium sulfate to release the soluble cytoskeletal elements. Dnase treatment at 25°C was used to remove the soluble chromatin. The remaining fractions

30 contained intermediate filaments and nuclear matrix proteins. This fraction was then disassembled with 8 M urea, and the insoluble components, which consisted

principally of carbohydrates and extracellular matrix components, were pelleted. The urea was dialyzed out, and the intermediate filaments were allowed to reassemble and removed by centrifugation. The nuclear

5   matrix proteins were then ethanol precipitated. All solutions contained freshly prepared 1 mM phenylmethyl-sulfonylfluoride (PMSF) to inhibit serine proteases, 0.3 μM aprotonin, 1 μM leupeptin and 1 μM pepstatin. Antibodies to proteins of this fraction were prepared

10   and demonstrated to be localized exclusively in the nucleus and isolated nuclear matrix fraction. The protein composition was determined by resuspending the proteins in 0.1 N sodium hydroxide and utilizing the Coomassie Plus protein assay reagent kit (Pierce,

15   Rockford, IL) with bovine serum albumin (BSA) as a standard.

        For two-dimensional gel electrophoresis, the ethanol precipitated NMPs were dissolved in a sample buffer consisting of 9 M urea, 65 mM 3-[(3-

20   Cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS), 2.2% ampholytes and 140 mM dithiothreitol. The final pellet containing NMPs represented less than 1% of the total cellular proteins.

25   High Resolution Two-Dimensional Electrophoresis

        High resolution two-dimensional gel electrophoresis was carried out utilizing the Investigator 2-D gel system (available from ESA Inc., Chelmsford, MA). Briefly, one-dimensional isoelectric

30   focusing was carried out for 18,000 V-h using 1-mm x 18-cm tube gels after 1.5 h of prefocusing. The tube gels were extruded and placed on top of 1-mm sodium dodecyl

sulfate Duracryl (available from ESA Inc., Chelmsford, MA) high tensile strength polyacrylamide electrophoresis slab gels, and the gels were electrophoresed with 12°C constant temperature regulation for approximately 5

5   hours. Gels were fixed with 50% methanol and 10% acetic acid. After thorough rinsing and rehydration, gels were treated with 5% glutaraldehyde and 5 mM dithiothreitol after buffering with 50 mM phosphate (pH 7.2). The gels were stained with silver stain (Accurate Chemical Co.,

10   Inc., Westbury, NY) or transferred to PVDF (Immobilon, Millipore Corporation) as follows. Fifty micrograms of nuclear matrix protein were loaded for each gel. Protein molecular weight standards were Silver Standards from Diversified Biotechnology (Newton Centre, MA).

15   Isoelectric points were determined using carbamylated standards from Gallard-Schlesinger, Inc. (Carle Place, NY) and Sigma Chemical Co. (St. Louis, MO). Multiple gels were run for each sample, and multiple samples run at different times. Only protein spots clearly and

20   reproducibly observed in all the gels of a sample type were counted as actually representing the nuclear matrix components. The gels were analyzed using the BioImage Electrophoresis Analysis System (BioImage, Ann Arbor, MI) which matches protein spots between gels and

25   databases the gels and protein spots.

The following NMPs were identified:

Table I

Proteins Associated With Human Bladder Cancer

5 | | Molecular Weight (kD) | pI |
|---|---|---|
| BLCA-1 | 72 | 7.70 |
| BLCA-2 | 40 | 7.50 |
| BLCA-3 | 39 | 6.27 |
| BLCA-4 | 37 | 6.24 |
| BLCA-5 | 29 | 5.80 |
| BLCA-6 | 22 | 8.00 |

Proteins Associated with Normal Human Bladder

15 | | Molecular Weight (kD) | pI |
|---|---|---|
| BLNL-1 | 70 | 6.09 |
| BLNL-2 | 66 | 5.84 |
| BLNL-3 | 66 | 5.80 |

The designation of each protein above is shown in the gels displayed in Figures 1-5. In addition, the following preliminary sequence data has been obtained: BLCA-1 includes the amino acid sequence LAKIVL (SEQ ID NO: 1). BLCA-4 includes the amino acid sequences EISQLNAG (SEQ ID NO: 2) and VYEDIMQK (SEQ ID NO: 3). BLCA-6 includes the amino acid sequence SLDLDLIIAEVK (SEQ ID NO: 4).

Production Of Antibodies

A standard protocol was followed in the production of antibodies against the BLCA-4 peptide of SEQ ID NO: 2. The peptide was modified slightly to include the addition of terminal cysteines for coupling purposes along with several amino acids for spacing to increase immunoreactivity. The sequence was verified through mass spectroscopy and conjugated to KLH. The peptide had the following structure:

BLCA-4   Acetyl-EISQLNAGAhxC-amide (SEQ ID NO: 2).

The peptide antigen was suspended in saline and emulsified by mixing with an equal volume of Freund's Adjuvant. Two New Zealand white rabbits (3-9 months

5   old) were injected with the peptide into three to four subcutaneous dorsal sites four times over a three month period. The animals were bled from the auricular artery and the serum collected from three production bleeds.

10   In order to assess the effectiveness of the immunization, the preimmune serum was tested at the same time as the production bleeds with an enzyme linked immunosorbent assay (ELISA) with BSA-coupled peptide on the solid phase. For all sera, ELISA

15   results were obtained by the detection with HRP-anti-Rabbit IgG conjugate and peroxidase dye at an OD492, all having a titering of greater than 1:10,000.

Testing The Antibody

20   The ability of the generated antibody to detect bladder cancer samples both by immunoblot and immunohistochemistry was demonstrated in the following manner. Bladder cancer samples and normal adjacent bladder tissue samples were taken from patients

25   undergoing cystectomies for bladder cancer. The antibody did not differentiate between the cancerous cells and normal cells from a patient with cancerous bladder cells. Next, samples were taken from normal bladders from individuals that did not have bladder

30   cancer. The antibody did not react with any of the samples that were examined.

The antibodies were then utilized to screen tissue

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and urine samples from individuals with and without
bladder cancer to determine the utility of this marker
to stratify individuals with bladder cancer from those
without the disease. BLCA-4 has been isolated and is
5   found in bladders of individuals with bladder cancer
but is absent from bladders from individuals without
this disease. In the bladder cancer patients, BLCA-4
is present throughout the bladder in both the tumor and
morphologically normal areas. In addition to the
10   detection of BLCA-4 in tissue samples, the present
inventors have also been able to demonstrate the
presence of BLCA-4 in urine samples of individuals with
bladder cancer.

Specifically, normal and tumor bladder tissue
15   samples from 24 patients undergoing surgery for bladder
cancer at the University of Pittsburgh Medical Center
were examined. All patients had transitional cell
carcinoma. The NMP composition of the 24 tumors and
their corresponding normal tissue were then analyzed,
20   utilizing a computer-based gel analysis system. The
nuclear matrix composition of all tumors was found to
express differences from the nuclear matrix composition
of the matched normal tissue from the same bladder.
Six proteins (BLCA-1 to BLCA-6) were identified that
25   were present in all of the tumors and were absent in
the adjacent normal tissue and three proteins that were
found in all of the normal bladder tissue samples and
were missing in the tumor samples. Tumors utilized in
these studies were of varying nuclear grades, including
30   nuclear grade 2 tumors. Nuclear grade 2 tumors do not
have a higher mitotic index than the normal bladder.
Therefore, the proteins that were identified do not

represent markers of proliferation. These differences appear to be unique to bladder cancer in that the molecular weights and isoelectric points of the proteins do not appear to correspond to those nuclear

5   matrix proteins previously reported to be different in prostate, renal, breast, head and neck or colon cancers. A number of normal tissue sites were examined where expression of these proteins was not found.

10 Immunoblot Analysis

The samples utilized for immunoblot analysis were from patients undergoing radical cystectomy at the University of Pittsburgh Medical Center. The patients ranged in age from 43-79 with a mean age of 61.8 (+/-

15 11). 38% of the sample population was female. In the immunoblot studies most of the patients have invasive TCC with grades of 3-4. Normal bladder tissues were obtained from organ donors who died of unrelated causes. Age ranges in the organ donor population was

20 19-67 with a mean age of 36.9 (+/- 19). In order to control for age differences, only samples from individuals greater than forty years of age were utilized in these studies. All samples were confirmed by the pathologist as containing either relatively pure

25 samples of tumor or normal cells.

Immunoassay

Urine samples from patients diagnosed with bladder cancer, along with normal controls, were collected and

30 tested with an enzyme linked immunosorbent assay (ELISA). The urine samples were precipitated in > cold, absolute ethanol (1:3) on ice (4°C) for 30

minutes. The samples were then centrifuged at 1877 x g at 4°C for 30 minutes. The pellet was suspended in appropriate volumes with phosphate buffered saline (1x). For coating the high binding 96-well plates, 50

5 ul each of sample was added to each well. Rabbit IgG was utilized as a positive control in these studies. The Rabbit IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) was diluted at 1:2000 with room temperature Tris buffered saline (25 mM Tris/HCl, 0.15

10 M NaCl) and incubated overnight at room temperature. The plates were rinsed three times with deionized water and blocked with 300 ul 1% BSA Blocking buffer in TBS (1% BSA, 1% non-fat powdered milk, 0.05% tween-20) for 30 minutes at room temperature. The washes were

15 repeated before treating the wells with antibody. The primary BLCA-4 anti-peptide antibody was added to the sample wells (50 ul/well), which was diluted at 1:10 in 2.5% BSA Blocking buffer in TBS (2.5% BSA, 2.5% non-fat powdered milk, 0.05% tween-20). Negative controls

20 consisting of normal rabbit serum (preimmune) diluted in 2.5% BSA Blocking buffer at 1:10, were added to another column and incubated 2 hours at room temperature. After the incubation, the washes were repeated. The secondary antibody was Goat-anti-rabbit

25 (Southern Biotechnology Associates, Inc., Birmingham, > AL) diluted at 1:1000 with 2.5% BSA Blocking buffer. To each well, except for the blank wells on the template, the secondary antibody (50 ul/well) was added and incubated for 2 hours at room temperature. The

30 plates were washed, wrapped in plastic wrap, and stored at 4°C overnight. To all the wells, including the blank, 75 ul of room temperature p-Nitrophenyl

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phosphate substrate (3mM p-Nitrophenyl Phosphate, 0.05M Sodium Carbonate, 0.05mM Magnesium Chloride) was added, incubated for 1 hour, and read at 405 nm. Statistical analysis was performed utilizing a two-sided Wilcoxson

5   signed rank test.

Results

The present inventors have been successful in utilizing the peptide sequence data for BLCA 1, 4 and 6

10 to raise antibodies against these peptides as well as to generate degenerate oligonucleotides with which to screen a cDNA library to obtain the entire cDNA sequences.

The two peptide sequences corresponding to regions

15 of the BLCA-4 protein have been analyzed. The first peptide (SEQ ID NO: 2) has a 75% homology with a number of non-vertebrate proteins including the Staph epidermis epiB protein and several yeast proteins. The second peptide of BLCA-4 (SEQ ID NO: 3) has 75%

20 homology with Arabidopsis thalianan ERECTA and lower levels of homology with other proteins in the BLAST database. In summary, it is not apparent from these two peptide sequences that BLCA-4 has any recognition with any known or characterized human proteins.

25 To date, positive BCLA-4 staining has been found in 100% of the "normal" tissues in the bladders of patients with bladder cancer. In the corresponding tumor samples, expression in 75% of the samples was found. There were several tumor samples that have low

30 levels of expression evident by further exposure of the blots but these values were not counted as being positive. When the bladders from unaffected

individuals were examined, expression was not found in any of the samples examined, even if these blots are over-exposed. The association between BLCA-4 and disease states was highly significant ($p < 2 \times 10^{-4}$,

5   Fisher exact two-sided test). This evidence demonstrates that the anti-BLCA-4 antibody is able to separate individuals with bladder cancer from those without disease. Further, these data support the presence of a field effect in the bladder, suggesting

10   that even cells that appear to be morphologically normal, have undergone alterations. The anti-BLCA-4 antibody is able to identify "normal" tissues in every patient with bladder cancer. It is therefore believed that this antibody can detect very early lesions of

15   bladder cancer, even prior to morphological alterations.

     Utilizing an ELISA, BLCA-4 was detected in the urine of patients with bladder cancer. Although the sample number was limited, BLCA-4 was detected in the

20   urine at levels significantly higher in patients with bladder cancer than without ($p = 2.4 \times 10-6$). The average value for BLCA-4 in the urine of normal control individuals is 3.5 +/- 3.7 whereas the average value for individuals with bladder cancer is 36.39 +/- 21.8.

25   Utilizing a cutoff value of 13 O.D. units, individuals with bladder cancer are distinguished from those without disease (Figure 1).

     A similar type of study was performed with patients with spinal cord injury. Bladder tumors are

30   known to occur up to 460 times more commonly in patients with spinal cord injuries (SCI) as compared to the general population. Bejany et al., J. Urol. 138:

1390-1392 (1987). The overall incidence of bladder cancer in this group of patients has been reported to be between 2.3 and 10%, Kaufman *et al.*, *J. Urol.* 118: 967-971 (1977), Melzak, *Paraplegia* 4: 85-96 (1966), accounting for 0.3% to 2.8% of all deaths in patients with SCI. Nyquist and Bors, *Paraplegia* 5:22-48 (1967), El-Masri and Fellows, *Paraplegia* 19: 265-270 (1981), Geisler *et al.*, *Paraplegia* 14: 262-275 (1977), Hackler, *J. Urol.* 117: 486-488 (1977). Of particular concern, there is a calculated 20 fold increased risk of death from bladder cancer in this population. Nyquist and Bors, *Paraplegia* 5: 22-48 (1967). Bladder tumors also appear to occur at an earlier age in patients with SCI as compared to the general population with up to 24% of all cases being detected in patients <40 years of age. Nyquist and Bors, *Paraplegia* 5: 22-48 (1967).

Utilizing one of the anti-BLCA-4 antibodies, described above, a large number of patients are being screened that are seen in the Spinal Cord Clinics at the Oakland VAMC, Pittsburgh, PA and at the Harmarville Rehabilitation Center, Pittsburgh, PA.

The study populations consists of approximately 600 patients with spinal cord injuries over a two year period. Urine samples are being collected from each individual and blindly analyzed for bladder cancer NMP levels utilizing the parameters previously described, the technician performing the assays has no knowledge of the source of the urine sample. As described above, the individuals with spinal cord injuries that have indwelling catheters receive annual cystoscopy and urine cytology. This yearly examination provides the opportunity to investigate the utility of this assay by

determining in the entire group individuals with
detectable bladder cancer. In addition, more frequent
cystoscopy will be performed if deemed appropriate.
The urologists will carefully analyze the bladders of
5   these individuals and detect and biopsy any suspicious
lesions in the bladder. The cystoscopic and urine
cytologic reports will be blinded by the urologist in
such a fashion that no patient identifiers will be
evident. They will be numbered in to coordinate with
10   the urine samples so that results from the nuclear
matrix analysis will be able to be correlated with
positive cystoscopic and cytologic examinations.

    These studies were started on April 1, 1998 and
have accrued 63 individuals with SCI. Utilizing our
15   O.D. cutoff value previously established (value >13),
12 individuals have been identified that are positive
based on the BLCA-4 assays (Figure 2).

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     While the invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and scope of the invention.